bosphore®

# Prothrombin Detection Kit v1

# USER MANUAL

For *in vitro* Diagnostic Use

Anatolia geneworks®

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## 1. PRODUCT DESCRIPTION

Bosphore® Prothrombin Detection Kit v1 detects Factor II mutation in the 3'UTR region of FII gene; namely G20210A (Prothrombin mutation/ a change of guanine to adenine) in human biological samples. Wild type FII gene is amplified and fluorescence detection is accomplished using the FAM filter. FII mutation is amplified and fluorescence detection is accomplished using the Cy-5 filter.

## 2. CONTENT

Bosphore® Prothrombin Detection Kit v1 is composed of Real-Time PCR reagents.

| Component | REAGENT | 100 Tests | 50 Tests | 25 Tests |
|---|---|---|---|---|
| 1 | $dH_2O$ | (1000 µl) | (1000 µl) | (1000 µl) |
| 2 | PCR Mix | (1375 µl) | (688 µl) | (344 µl) |
| 3 | Detection Mix1 | (100 µl) | (50 µl) | (25 µl) |
| 4 | Positive Control | (88 µl) | (44 µl) | (22 µl) |

## 3. STORAGE

Bosphore® Prothrombin Detection Kit v1 PCR reagents should be stored at -20°C. Repeated thawing and freezing (>3x) should be avoided since it may reduce sensitivity. If the components are to be used in small amounts, they should be frozen in aliquots.

While preparing the PCR; the components should not be exposed to room temperature for more than 10 min and the detection mix components should not be exposed to light more than 1-2 min. We recommend preparing the PCR on a cooling block and keeping the detection mixes within a closed container.

The components maintain their stability until the expiry dates on the labels, if they are stored at advised conditions.

## 4. REQUIRED MATERIALS AND DEVICES

- Montania® 483 Real-Time PCR Instrument (Anatolia Geneworks), or another Real-Time PCR system with FAM and Cy5 filters (iCycler, iQ5, CFX–BioRad, LightCycler 1.5, 2.0, 480-Roche, 7500 Real-Time PCR System-ABI, Stratagene Mx3005P, Mx3000P-Agilent, LineGeneK, LineGene 9600-Bioer, Rotorgene 2000, 3000, 6000, Q-Qiagen)
- 0.2 ml Thin-Wall PCR tubes or strips
- Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit (Anatolia Geneworks),or other high quality genomic DNA extraction kits/systems
- Deep freezer (-20°C)

- Desktop centrifuge with rotor for 2 ml. microcentrifuge tubes
- Calibrated adjustable micropipettes
- DNAse, RNAse, pyrogen free micropipette tips with filters
- DNAse, RNAse, pyrogen free 1.5 or 2 ml. microcentrifuge tubes
- Disposable laboratory gloves

## 5. IMPORTANT NOTES AND SAFETY INSTRUCTIONS

**Important!:**

- The product should be delivered on dry ice. Check for presence of dry ice upon arrival.
- Check for the expiry dates on the box and tube labels, upon arrival. Do not use expired products or components.
- Calibrated or verified micropipettes, DNAse, RNAse, pyrogen free micropipette tips with filters, and DNAse, RNAse, pyrogen free microcentrifuge tubes should be used.
- Before starting a test procedure, all components should be thoroughly thawed. After thawing, all components should be centrifuged briefly (spin-down for 3-5 seconds), and mixed well to ensure homogeneity prior to use.
- The kit components should be kept on ice or a cooling block until the reaction is prepared, and they should be quickly returned to -20°C.
- PCR and nucleic acid isolation must be performed in different compartments. Samples should be stored separately to avoid contact with the kit components.
- All the biological wastes produced during the nucleic acid isolation step; including the blood samples and material contacted with them, should be discarded into medical waste and disposed safely.

## 6. PRODUCT USE LIMITATIONS

- All the components may exclusively be used for in vitro diagnostics.
- This product should be used in accordance with this user manual.
- This product is to be used by personnel specially trained to perform in vitro diagnostic procedures.

## 7. MUTATION

Prothrombin which is also called Factor II is a protein in the blood involved in the blood clotting mechanism. It is needed to form fibrin which is then required for blood clotting. Recently, a new polymorphism in the 3'-untranslated region of the Prothrombin gene (F2), guanine 20210

adenine mutation is described which alters the polyadenylation site of the gene resulting in excess mRNA synthesis with subsequent increased protein expression. That is found to be associated with elevated levels of prothrombin and results in increased risk for hypercoagulability disorders (Thrombophilia). [1], [2], [3]

The G20210A is the second more frequent variant implicated in hypercoagulability disorders of the caucasian population and has the prevelance of approximately 1 to 4% in the normal population. İt is more prevalant in southern European and rarely seen in Asians or Africas.[4] A study of patients in Turkey revealed the presence of the prothrombin 20210a mutation in 0.7% of subjects.[5] The G20210A variant, an autosomal recessive deficiency, which is usually not accompanied by other factor mutations ( i.e. factor V-Leiden) may be inherited heterozygous (1 pair), or rarely, homozygous (2 pairs), and not mainly related to gender or blood type. Also with other risk factors; including congenital thrombophilia (deficiency of protein C and protein S...etc.), temporary factors such as surgery, immobility, pregnancy and chronic conditions such as obesity and cancer, homozygous mutations increase the risk of thrombosis more than heterozygous mutations.[6]

## 8. METHOD

Bosphore® Prothrombin Detection Kit v1 is based on the Real-Time PCR method. Polymerase chain reaction is a technique that is used for amplification of a DNA region. The reaction occurs by the repeating cycles of heating and cooling. The main components of PCR are primers, dNTPs, Taq polymerase enzyme, buffer solution and template. Primers are small synthetic DNA those anneal to the specific regions of the template in order to start the synthesis. dNTPs are the building blocks of the amplified products. Taq polymerase amplifies the DNA template. Buffer solution provides the pH adjustment required for the reaction and template, as referred, is the target region for synthesis.

In Real Time PCR technique, in contrast to conventional PCR, PCR product can be monitored during the reaction. Therefore, Real-Time PCR obviates the need for further analysis methods like gel electrophoresis, whereby minimizing the risk of contamination. Dual-labeled probes employed in the reaction in addition to the conventional PCR reagents, enable detection of the amplified target with increased sensitivity.

The test utilizes the 5′ exonuclease activity of Taq Polymerase to cleave a dual-labeled fluorescent hydrolysis probe during the extension phase. The probe is labeled at the 5′ end with a fluorescent reporter, and at the 3′end with another fluorescent molecule that acts as a quencher for the reporter. When the two fluorophores are in close proximity, even if the reporter is excited by light, no reporter fluorescence can be detected. During the elongation step, Taq Polymerase

encounters and cleaves the probe bound to the template. As the reporter is relieved from the suppressing effect of the quencher, fluorescence signal can be detected.

As the PCR product accumulates, the fluorescence generated by the reporter increases. The point at which the signal rises above background level and becomes detectable, is called the threshold cycle ($C_T$).

Bosphore® Prothrombin Detection Kit v1 employs multiplex PCR. FII DNA whether wild-type or mutant, is amplified in a single reaction, using sequence-specific primers against mutant and wild-type alleles. The fluorescent signal generated by the mutant-type FII gene amplification is detected by a probe labeled at the 3' end with FAM, through the FAM channel. In contrast, the fluorescent signal generated by the wild-type allele amplification, is detected by a second probe (labeled at the 5' end with a different reporter molecule, Cy5) through the Cy5 channel.

## 9. PROCEDURE

### 9.1. Sample Preparation, Storage and Transport

To isolate serum from the clinical specimen, the blood sample should be collected into sterile vacutainers without any anticoagulant. For venipuncture, only sterile material should be used. The serum should be separated from blood within 6 hours after blood collection. To separate the serum, the blood container should be centrifuged at 800-1600 x g for 20 minutes. The separated serum should be transferred to polypropylene tubes and stored at -20ºC or lower, until use. The samples should be transported in containers with capacity to resist pressure.

### 9.2. Interfering Substances

To avoid possible influences on PCR:

- Samples which have been collected in tubes that contain anticoagulants such as heparin or EDTA,
- Hemolytic samples,
- Samples of heparinised patients,
- Samples of patients with elevated levels of bile salts, bilirubin or lipids

must not be used.

### 9.3. DNA Isolation

We recommend that the Magnesia® 16 Nucleic Acid Extraction System / Magnesia® Genomic DNA Whole Blood Kit (Anatolia Geneworks) isolation system is used with Bosphore® PROTHROMBIN Detection Kit v1. The DNA isolation should be performed according to the manufacturers' instructions. The starting volume is 400 µl; the elution volume is 60 µl.

### 9.4. Kit Components

#### 9.4.1. PCR Mix

HotStarTaq DNA Polymerase: HotStarTaq DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into E.Coli. The enzyme is provided in an inactive form. It is activated by a 15-minute 95 °C incubation step. This prevents the formation of misprimed products and primer-dimers during reaction setup and the first denaturation step, leading to high PCR specificity and accurate quantification. PCR Buffer: contains Tris-Cl, KCl, $(NH_4)_2SO_4$, 8 mM $MgCl_2$, pH 8.7 (20°C ). dNTP Mix: Contains ultrapure quality dATP, dGTP, dCTP ve dTTP/dUTP.

#### 9.4.2. Detection Mix 1

Detection Mix 1 contains F II gene -specific forward and reverse primers and two dual-labeled probes against wild-type and mutant F II allele.

#### 9.4.3. Positive Control

The positive control contains heterozygous FII gene containing one copy of the wild-type and mutant FII allele each. It should be included in the PCR to efficiently test whether the samples being analyzed are positive or negative. The threshold cycle for the positive control is given in the acceptance criteria table (Section 10. Analysis). Threshold cycles higher than the acceptance criteria may indicate an efficiency loss in the reaction.

### 9.5. Preparing the PCR

Positive control should be added into the PCR reaction together with the samples and the negative control (PCR-grade water). Make sure that all the kit components are thawed before use. Refer to the table below for preparing the PCR. It is for only one reaction, multiply these values with the sample number to find the values required for the master mix. While preparing master mixes for more than 3 samples, an extra 10% should be added to the total sample number.

| | |
|---|---|
| **PCR Mix** | 12.5 µl |
| **Detection Mix 1** | 0.9 µl |
| **dH2O** | 6.6 µl |
| **Sample DNA** | |
| **Positive/Negative Control** | 5 µl |
| | |
| **Toplam Hacim** | 25.0 µl |

Pipette 20 µl of the master mix into the PCR tubes or strips, and add 5 µl of DNA (sample/positive or negative control). Close the tube cap. Make sure that the solution in each tube is at the bottom of the tube. Centrifuge if necessary.

### 9.6. Programming the Montania® 483 Real-Time PCR Instrument

The thermal protocol for Bosphore® PROTHROMBIN Detection Kit v1 is composed of an initial denaturation for activation the HotStarTaq DNA Polymerase, a two-step amplification cycle and a terminal hold. The real-time data is collected at the second step of the amplification cycle.

| | | | |
|---|---|---|---|
| Initial denaturation | 95°C | 14:30 min. | |
| Denaturation | 95°C | 00:30 min. | 40 cycles |
| Annealing and Synthesis (Data Collection) | 63.5°C | 00:30 min. | |
| Hold | 22°C | 05:00 min. | |

Montania® 483 Real-Time PCR Instrument is installed and calibrated as it is delivered to the end user.

In order to establish an appropriate link between the system components, first the thermal cycler and the optical module, and then the PC and the software should be started.

Before starting a Real-Time PCR reaction using the Bosphore® Kits, the following steps should be completed:

- Choose the filter pairs to be used (FAM and Cy5),
- Identify unknown samples, positive and negative controls,
- Select the correct thermal protocol.

These steps are described below:

From the main menu of the Montania® 483 Real-Time PCR Instrument, “File” and then “New” is selected. “Create a new Experiment” is selected. In the “Select Channel” window channels 1 (FAM) and 3 (Cy5) are selected (Fig. 1). Samples, positive and negative controls are identified in the “Module Edit” menu (Fig. 2). To select the thermal protocol “Gene Amplification” menu is used. The “Open” button in the “Experiment Program” is clicked and the appropriate thermal protocol is selected. (Fig. 3a). The thermal cycles of the selected protocol is displayed. The experiment starts by clicking the “Start” button (Fig. 3b).

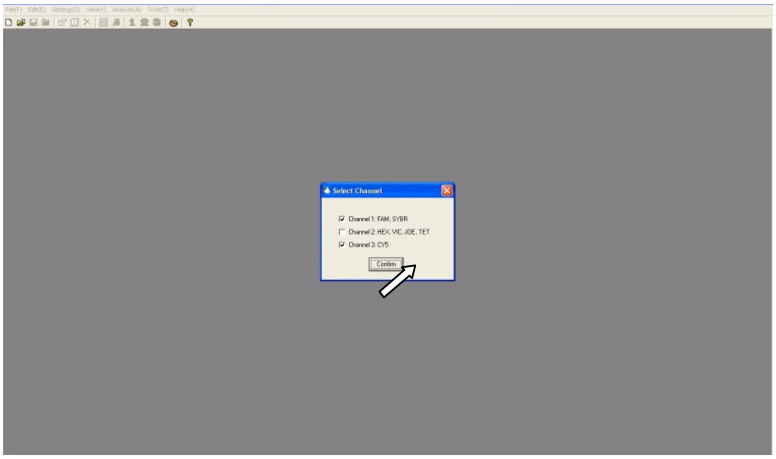


Fig. 1: Filter Selection in Montania® 483

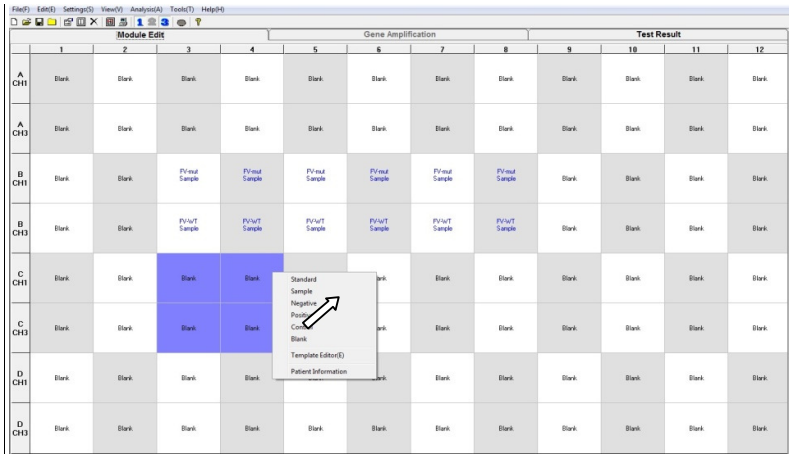


Fig. 2: Sample Location and Identification

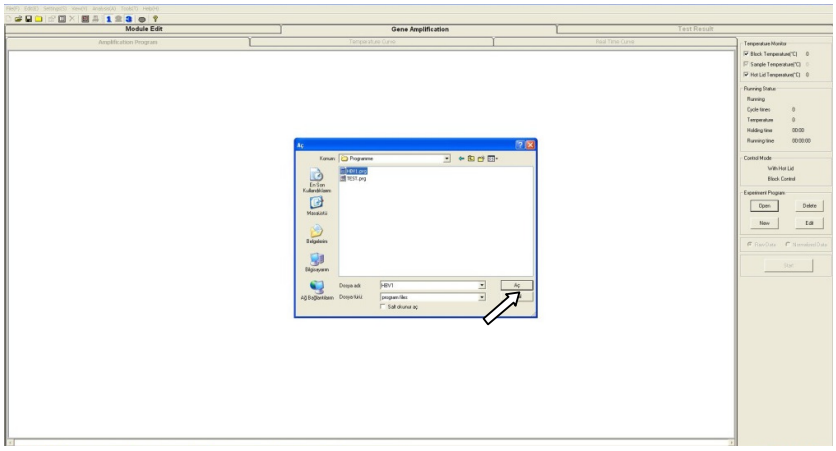


Fig. 3a: Selecting the Thermal Protocol

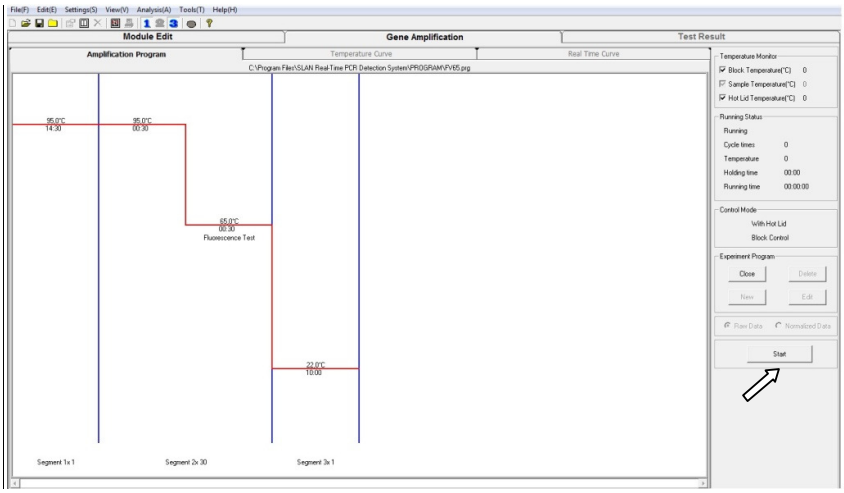


Fig. 3b: Starting the Experiment

## 10. ANALYSIS

By the end of the thermal protocol, the Montania® 483 Real-Time PCR Instrument software automatically calculates the baseline cycles and the threshold.

Example of an amplification curve is given in Fig. 4.

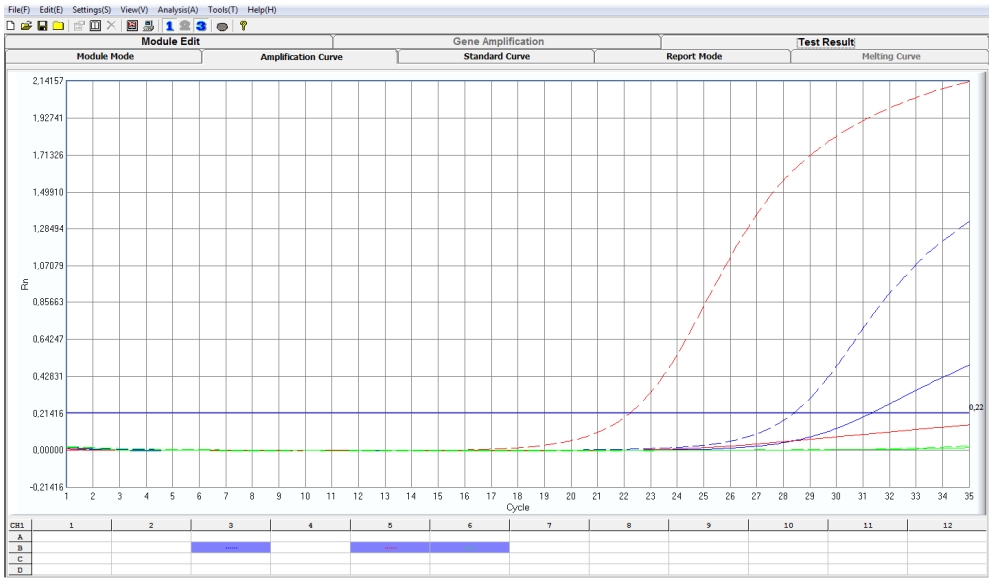


Fig. 4: Amplification Curve of a Bosphore® PROTHROMBIN Detection Kit v1

Analysis of the results should be performed by trained personnel who have received the required training for analysing Real-Time PCR data. We recommend that the test results must be

evaluated by an expert clinician, taking the patient's clinical findings and the results of other tests into consideration.

All analysis is done automatically in routine use. However, when the trained personnel, who have received the required training from manufacturer, consider it as necessary, the system allows pulling down the threshold as much as possible in order to detect slight amplifications. In this case, attention should be paid to keep the threshold line above the background.

Amplification should be observed from both FAM and Cy5 filters of the heterozygous positive control during the test. The amplification of both filters from the tested samples should be compared with that of the positive control. Test results should not be reported unless the assay results meet the criteria stated above. Please contact the manufacturer if an impairment in the product's performance is observed (See the last page for contact information).

The qualitative results of the test are displayed on the "Report Mode" screen. The samples that cross the threshold in Channel 1 (FAM) and Channel 3 are displayed as positive, for wild-type and mutant alleles respectively.

The following table shows the possible results and their interpretation:

| | |
|---|---|
| Signal in both FAM and Cy5 | Heterozygous mutant: The sample has both wild-type and mutant FII alleles. |
| No signal in FAM, signal in Cy5 | Homozygous wild-type: The sample has only wild-type FII DNA |
| Signal detected only in the FAM filter | Homozygous mutant: The FII DNA in the sample is composed of mutant alleles |
| No signal in FAM and Cy5 | The test should be repeated |

## 11. TROUBLESHOOTING

Please contact the manufacturer in case of a problem during a run.

**Signal from FAM/Cy5 Filter in the Negative Control**

| | |
|---|---|
| Contamination | Use filter-tips. Repeat PCR with new kit components. |

**The Threshold is Above Low Signals**

| | |
|---|---|
| The threshold should be manually adjusted | Using the mouse pull the threshold down until it cuts the low signals. Avoid the background and the signal from negative control. |

## 12. REFERENCES

1. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood. Nov 15 1996;88(10):3698-703

2. Pollak ES, Lam HS, Russell JE. The G20210A mutation does not affect the stability of prothrombin mRNA in vivo. Blood. Jul 1 2002;100(1):359-62
3. Ceelie H, Spaargaren-van Riel CC, Bertina RM, Vos HL. G20210A is a functional mutation in the prothrombin gene; effect on protein levels and 3'-end formation. J Thromb Haemost. Jan 2004;2(1):119-27
4. Rosendaal FR, Doggen CJ, Zivelin A, et al. Geographic distribution of the 20210 G to A prothrombin variant. Thromb Haemost. Apr 1998;79(4):706-8
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6. DeStefano V, Martinelli I, Mannucci P, et al. The risk of recurrent deep venous thromboembolism among heterozygous carriers of the G20210A prothrombin gene mutation. Br J Hematol. 2001;113:630–635.

## 13. SYMBOLS


Use by

Lot/Batch

Catalog number


Temperature limitation


Caution, consult accompanying documents


Manufacturer

In Vitro Diagnostic Medical Device

## 14. CONTACT INFORMATION

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